Short communication

Isolation of punicalagin from *Punica granatum* rind extract using mass-directed semi-preparative ESI-AP single quadrupole LC-MS

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Abstract

We are living in a era of alarming increases in microbial resistance to currently available antibiotics, and there is a growing need for new pharmaceutical products to treat infectious diseases. The pomegranate is an edible fruit that has virucidal and antimicrobial activities which is primarily attributable to the high concentration of hydrolysable tannins. Punicalagin, a high molecular weight tannin (1084.7), accounts for approximately 70% of the total and is concentrated in the fruit exocarp (rind). It is the focus of much research, although it is prohibitively expensive to purchase which presents an obstacle to further exploitation and development. Here we describe a method for the isolation of punicalagin from pomegranate rind extract and total pomegranate tannins using an Agilent preparative mass-directed LC-MS single quadrupole ESI-API system, where the ionization was set as negative and the mass signal acquired in Single Ion Monitoring. Thanks to the automatic fraction collector, the method can be used in automatic mode and is capable of producing punicalagin with >95% purity.

Keywords: Punica Granatum, Pomegranate Rind Extract, TPT, Punicalagin, LC-MS

1. Introduction

We are currently living in an era of alarming and unprecedented increases in microbial resistance to our current arsenal of antibiotic drugs, and no new drugs have come onto the market in 30 years or so. New products are thus urgently needed [1, 2]. In addition, there is a clear predilection by patients for medicines that are of natural origin compared to synthetic drugs [3, 4].

The fruit of *Punica granatum* L. (pomegranate) has been used as a healthy and healing fruit for many years. It has long been used in Middle Eastern and Chinese medicine for its antioxidant, anti-inflammatory [5] bactericidal [6] and virucidal [7] properties. The phytochemicals constituents of pomegranates include many chemical species including sugars, flavonoids, anthocyanin, tannins, [8] and can be obtained from most parts of the fruit but the exocarp, or rind, has been shown to contain the highest concentration of polyphenols. A major class of compounds is the hydrolysable ellagitannins of which punicalagin (2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose) constitutes up to 70% [Figure 1]. It is a large molecule (MW 1084.71) and is comprised of 3 moieties: gallagic acid and ellagic acid linked via a glucose unit. The molecule naturally adopts one of two anomeric forms, α and β, depending on whether the glucose is in the boat or chair conformation, and the two exist in a 1:2 ratio under standard conditions.

Ongoing research shows that punicalagin is primarily responsible for antimicrobial, anti-inflammatory and virucidal properties of PRE. The microbicidal activities of PRE and punicalagin have been shown to undergo significant potentiation when co-administered with metal ions, eg Zn (II) [9, 10]. Punicalagin therefore has potential as a therapeutic agent as outlined; however, it is very expensive to purchase (eg £217 for 10 milligrams from one major supplier) which presents a barrier to further development, as substantial quantities are required to carry out the necessary pre-clinical drug development work.

PRE is typically prepared from pomegranate rinds by extraction into boiling water [9] followed by centrifugation and filtration. The non-tannin components of PRE can be removed by column chromatography to yield concentrated total pomegranate tannins (TPT), for example using Amberlite XAD-16 resin. Punicalagin may then be isolated by column chromatography using a Sephadex LH-20 column which entails a complex gradient elution [11] or by countercurrent chromatography [12]. The methods typically used to isolate punicalagin thus require numerous time-consuming laboratory steps and lengthy sample preparation.

Given the need for new antimicrobial molecules has arisen over recent years, fast and reproducible methods to isolate significant amounts of antimicrobial molecules has the potential to expedite the development of new therapeutic products. Mass-directed purification is a type of preparative LCMS in which the instrument may be set to collect column eluate upon detecting a pre-selected mass or mass range, once the chromatographic conditions have been established. These sophisticated instruments are available from a number of manufacturers including Waters and Shimadzu, although we have in our laboratory an Agilent 1260 Infinity Automated LC/MS Purification System [13]. In the current work we describe a method for the isolation of high-purity punicalagin using this system.

2. Materials and methods

2. 1 Materials

Amberlite XAD-16 (Alfa Aesar), LCMS-grade water and methanol were purchased from Fisher Scientific, Loughborough, UK. Pomegranates, of Spanish origin, were purchased from a local store.

2.2 Preparation of Pomegranate Rind Extract (PRE)

The rinds from five pomegranates were cut in small pieces of approx. 2cm and blended in deionized water, 25% w/v, before being boiled for 10 min and transferred to 50mL tubes and centrifuged 7 times at 5840rpm for 30 min on each occasion. The supernatant was then vacuum filtered through 0.45µm nylon membranes (Whatman, UK) to yield a clear dark yellow-orange solution which was then freeze-dried and stored at -20°C until required [14].

2.3 Preparation of Total Pomegranate Tannins (TPT) from PRE

Based on a previously reported method [11] a glass column was slurry-packed with 50g of Amberlite XAD-16 resin in degassed and deionized water. This was then washed with methanol, followed by deionized water, and then was left overnight to equilibrate. A 2cm layer of sand was added to the top and the bottom. Freeze-dried PRE was reconstituted in deionized water at a concentration of 200 mg/mL and loaded onto the column. Firstly, the water-soluble components were washed out using a copious amount of water, then the TPT fraction was eluted after washing with 70mL of methanol and collected. This solution was then rotary evaporated using a Buchi instrument with the temperature set at 40°C and the vacuum at 50mbar. The TPT product was stored at 2-4 °C until further use. Analysis of the tannin-free fraction (section 2.4) confirmed that no tannins were present (data not shown).

2.4 Analytical HPLC analysis of TPT

The TPT was analysed using an Agilent 1100 system fitted with a Kinetex C18 150 x 4.6mm 5µm 100Å RP column (Phenomenex, Macclesfield, UK). Based on a previous method [9] a gradient mobile phase was used composed of: solvent A H_2O + 0.1%TFA and solvent B: MeOH + 0.1%TFA. The gradient timetable started with 5%B, 0-3 min 10%B, 3-15 min 20%B, 15-20 min 40%B, 20-25 min 60%B, 25-27 min 5%B, 27-

35 min 5%B. A 1 mg/mL sample in deionized H_2O was prepared, the injection volume was 20 µL and the detection was performed at a wavelength of 258 nm with a UV detector.

2.5. Semi-preparative Isolation of Punicalagin using an LC-MS system

The isolation was performed using a modular semi-preparative LC-MS ESI-AP single quadrupole system with automatic fraction collector (Agilent Technologies, Stockport, UK). Waters X-Select CSH C18 OBD 5µm 130Å columns were used: firstly a 50 x 10mm scout column which was used to set up a reliable extraction method, then a 250 x 19mm semi-preparative column. The solvents used were H_2O (channel A) and MeOH (channel B); no mobile phase acidification was used. The separation was performed in Single Ion Monitoring (SIM) negative ionization mode, with a capillary voltage of 4000V, drying gas temperature of 350°C and gas nebulizer pressure of 32 psi [15]. The threshold of the automatic fraction collector was set at 2000.

The gradient was scaled from the analytical method to be reproducible with these columns. Firstly, the scout column method was converted from the analytical one, and a run was performed to check the reliability of the separation. The sample was 100mg/mL in H_2O and 300µL were injected. Further runs were carried out to control the consistency of the separations. The gradient used with this column started at t_0 with 5%B, 0-0.38 min 10%B, 0.38-1.80 min 20%B, 1.80-2-39 min 40%B, 2.39-2.98 min 60%B, 2.98-3.22 min 5%B, 3.22-4.16 min 5%B. Although even with this column was possible to isolate punicalagin, only low amounts were collected, so the method evolved to the big semi-preparative column. The gradient for this column started with 5%B, 0-7.77 min 10%B, 7.77-33-36 min 20%B, 33.36-44.02 min 40%B, 44.02-54.69 min 60%B, 54.69-58.95 min 5%B, 58.95-76.01 min 5%B. This sample load was 225mg in 1.5mL H2O. The flow rate for both the columns was 20mL/min. Both manual and automatic collection were performed. As already stated above, the threshold for the automatic collection was set above 2000. The samples obtained were then dried to yield the purified punicalagin; firstly, the methanolic fraction was evaporated using a Buchi rotary evaporator (50 mbar, 50 °C), then the remaining solvent (aqueous) was removed by freeze-drying.

3. Results and discussions

As can be seen in the analytical HPLC chromatogram [Figure 2], the two anomers of punicalagin were well separated in the preparative LCMS method described. The two main peaks α and β punicalagin eluted with a different concentration of solvent B and always tend to an approximate ratio of 1:2 ratio. The peak at the end can be identified as ellagic acid, one of the major building blocks of punicalagin. The analysis of the TPT was performed to prove the presence of punicalagin, so it was possible to proceed with the purification. Because of the differences in the stationary phase between the preparative and the analytical columns, the

chromatogram of the preparative HPLC doesn't appear well separated, but thanks to the mass spectrometric collection triggering, it was still possible to isolate highly pure punicalagin. Figure 2 shows the plot of the run with the small scout column. The vertical lines show the windows in which the punicalagin (alpha the first and beta the second) was collected; the ratio of the two isomers was found also in the mass chromatogram.

The analytical resolution of punicalagin utilises acidification, typically by trifluoroacetic acid (TFA) [9]. In LCMS the preferred acidifying reagent is formic acid as, compared to TFA, it tends not to leave behind residual molecules. However, we found that when the punicalagin fractions were evaporated to dryness, it was discovered that nearly all the punicalagin had degraded. This was attributed to the increasing acidity that accompanied drying as the acid was also increasing in concentration. Consequently, it was decided to omit altogether the formic acid to ensure greater stability of the punicalain, albeit at the expense of superior chromatographic resolution.

The semi-preparative column report [Figure 3] shows a different HPLC chromatogram and a similar SIM chromatogram, probably due to the different sample loading in the two columns: in the small scout column it was closer to the optimal loading. In this case, even though both the little signals in the mass spectrum were slightly above 2000, the only fraction collected was the high peak at 22 min. For completeness, each fraction collected was re-run through the preparative LC-MS [Figure 4] to check the purity of the punicalagin, and all the signals detected were approximately the same: the differences represented the different grade of purity (average of >95% pure). These tests were performed after 1 hour.

The purity of the collected punicalagin product was determined by analytical HPLC [9]. Figure 5 shows that 95% of the integrated areas (compared to the total areas) was accounted for by the punicalagin anomer peaks. Interestingly, we attempted to collect individual anomers separately, by programming the instrument to collect at times corresponding to α and β punicalagin accordingly - it would be of interest to determine if the anomers give rise to differing biological (eg anti-microbial, anti-inflammatory) activities. However, even though collections were seemingly successful using the acid-free elution, it was noted that each 'purified' anomer reverted to the 1:2 ratio after drying, with β-punicalagin is the more abundant anomer at neutral pH [16].

4. **Conclusions**

A rapid, reliable and automatable method to isolate punicalagin from TPT was developed using the 1260 Infinity Automated LC/MS purification system. This facilitating the development work in order to bring to market new punicalagin-based antimicrobial products at a time of great need whilst avoiding large expenses to purchase it from suppliers. Work is ongoing to purify punicalagin directly from PRE, thus avoiding the prior concentration of TPT through Amberlite. However, the TPT separation step is not onerous, and does provide a material that is freely soluble in methanol, useful for easy injection into the LCMS.

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Figures:

1. Chemical structure of punicalagin
2. HPLC chromatogram of PRE
3. LC-MS report of TPT using the Scout column
4. LC-MS report of TPT using the Semi-preparative column
5. LC-MS report of collected pure punicalagin